Journal of Thrombosis and Haemostasis, **8**: 1302–1312 DOI: 10.1111/j.1538-7836.2010.03816.x

ORIGINAL ARTICLE

Generation and characterization of inhibitory nanobodies towards thrombin activatable fibrinolysis inhibitor

K. BUELENS,* G. HASSANZADEH-GHASSABEH,†‡ S. MUYLDERMANS,†‡ A. GILS* and P. J. DECLERCK*

**Laboratory for Pharmaceutical Biology, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, Leuven; †Laboratory of Cellular and Molecular Immunology, Faculty of Sciences and Bio-Engineering Sciences; and ‡Department of Molecular and Cellular Interactions, Vrije Universiteit Brussel, Brussels, Belgium*

To cite this article: Buelens K, Hassanzadeh-Ghassabeh G, Muyldermans S, Gils A, Declerck PJ. Generation and characterization of inhibitory nanobodies towards thrombin activatable fibrinolysis inhibitor. *J Thromb Haemost* 2010; **8**: 1302–12.

Summary. *Background and objective*: As activated thrombin-activatable fibrinolysis inhibitor (TAFIa) is a potent antifibrinolytic enzyme, the development of TAFI inhibitors is a new promising approach in the development of profibrinolytic drugs. We, therefore, aimed to generate nanobodies, camelid-derived single-domain antibodies towards TAFI. *Methods and results:* This study reports the generation and characterization of a panel of 22 inhibitory nanobodies. This panel represents a wide diversity in mechanisms for interference with the functional properties of TAFI as the nanobodies interfere with various modes of TAFI activation, TAFIa activity and/or TAFI zymogen activity. Nanobodies inhibiting TAFIa activity and thrombin/thrombomodulin-mediated TAFI activation revealed profibrinolytic properties in a clot lysis experiment with exogenously added thrombomodulin (TM), whereas nanobodies inhibiting plasmin-mediated TAFI activation only revealed profibrinolytic properties in a clot lysis experiment without TM. The results of *in vitro* clot lysis experiments provided evidence that inhibitory nanobodies penetrate the clot better compared with inhibitory monoclonal antibodies. *Conclusions:* These data suggest that the generated nanobodies are potent TAFI inhibitors and are a step forward in the development of a profibrinolytic drug. They might also be an excellent tool to unravel the role of the physiological activators of TAFI in various pathophysiological processes.

Keywords: fibrinolysis, inhibition, nanobodies, TAFI, profibrinolytic.

Correspondence: Paul Declerck, Laboratory for Pharmaceutical Biology, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, Campus Gasthuisberg, O&N2, PB 824, Herestraat 49, B-3000 Leuven, Belgium.
Tel.: +32 16 32 34 31; fax: +32 16 32 34 60.
E-mail: paul.declerck@pharm.kuleuven.be

Received 19 August 2009, accepted 13 February 2010

Introduction

Thrombin activatable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase U (proCPU), forms a link between coagulation and fibrinolysis. TAFI is synthesized by the liver and circulates in plasma as a zymogen of 56 kDa [1–3]. The zymogen is activated by thrombin, thrombin/thrombomodulin (T/TM) or plasmin, resulting in the activation peptide (AP; Phe^{1}-Arg^{92}; 20 kDa) and the catalytic domain TAFIa (Ala^{93}-Val^{401}; 36 kDa, CPU) [1,4,5]. TAFIa attenuates fibrinolysis by removing surface-exposed C-terminal lysine residues from fibrin, which are required for a positive feedback in plasmin generation [6]. This mechanism provides the rationale for inhibiting either the activation of TAFI or the TAFIa activity, in order to enhance clot lysis. Furthermore, elevated TAFI(a) levels are considered to be a risk factor for deep vein thrombosis, angina pectoris and coronary artery disease [7,8]. In addition, the broad substrate specificity of TAFIa (e.g. bradykinin, anaphylatoxines C3a and C5a and fibrin) leads to additional roles of TAFI in the modulation of blood pressure, cell migration and inflammation [9–12].

Previous studies demonstrated that co-infusion of a TAFIa inhibitor, isolated from the potato tuber (PTCI), enhanced the apparent potency of t-PA 3-fold in rabbit thrombosis models [13,14]. Similarly, in an arterio-venous shunt model of thrombosis in rat, thrombolysis was observed when a TAFIa inhibitor was added whereas under identical conditions, t-PA alone did not reduce the thrombus weight [15]. Likewise, cotreatment with a small-molecule TAFIa inhibitor, BX 528, enhanced the thrombolytic effects induced by exogenous t-PA in three animal models [16]. The same study also reported less bleeding complications upon co-administration of a TAFIa inhibitor compared with conventional therapy [16].

Even although TAFIa is a very potent antifibrinolytic enzyme, no physiological inhibitors are known. Nevertheless, TAFIa is regulated by its intrinsic, temperature-dependent instability (i.e. functional half-life of TAFIa is 5–15 min at 37 °C) [17]. Recently, a stable TAFI variant [i.e. TAFI-A^{147}-C^{305}-I^{325}-I^{329}-Y^{333}-Q^{335} (TAFI-ACIIYQ)] has been reported

with a 180-fold longer half-life and a 18-fold higher antifibrinolytic potential compared with TAFI-wt [18].

So far, several inhibitors of TAFIa have been characterized: chelating and reducing agents [3,19], arginine and lysine analogs [4,20] and naturally occurring metallocarboxypeptidase inhibitor isolated from potato (PTCI) [6]. Also small synthetic inhibitors have been developed [16,21–25]. To circumvent problems of specificity, monoclonal antibodies (mAbs) have been raised against TAFI [26–28]. Some of these mAbs interfere with the activation of TAFI whereas others interfere directly with the catalytic activity of TAFIa. However, therapy with murine antibodies encounters well-known problems of immunogenicity. Even although this can be circumvented by the generation of smaller derivatives such as single-chain variable fragments [29] or by humanization [30], these derivatives may encounter other difficulties such as reduced affinity [31], aggregation and proteolysis [32].

Serum of *Camelideae* contains an important fraction of functional antibodies that are naturally devoid of light chains, called heavy-chain antibodies [33]. These heavy-chain antibodies therefore recognize the antigen by a single variable-domain heavy-chain (termed VHH or Nanobody™). These nanobodies have many inherent, advantageous properties, such as their low molecular mass (15 kDa), low immunogenicity [34], high affinity, high solubility and stability [35] and easy production of the recombinant VHH in bacteria or yeast [36,37]. The hypervariable regions [i.e. complementary determining regions (CDR)] of nanobodies are on average longer than those of conventional antibodies, most probably to compensate for the loss of the binding regions on the light chain. Nanobodies are often potent enzyme inhibitors, due to their long CDR3 domain, that often forms an extended loop and therefore is able to penetrate into the catalytic cleft of enzymes [38]. Taken together, these characteristics led us to hypothesize that the generation and selection of nanobodies towards TAFI(a) might be a successful approach to develop potent TAFI(a) inhibitors.

Here we report the generation and characterization of a panel of 22 nanobodies raised towards TAFI and exhibiting inhibitory properties towards various modes of TAFI activation, TAFIa activity and/or TAFI zymogen activity thereby representing a high diversity in functional properties.

Materials and methods

Materials

Wild-type recombinant TAFI-T^{147}-I^{325} (TAFI-TI), one of the naturally occurring TAFI isoforms, TAFI-A^{147}-C^{305}-I^{325}-I^{329} (TAFI-ACII) and TAFI-A^{147}-C^{305}-I^{325}-I^{329}-Y^{333}-Q^{335} (TAFI-ACIIYQ) were prepared as described previously [26]. All experiments in this study were performed with TAFI-TI, unless otherwise indicated. Oligonucleotides used for cloning and sequencing were obtained from Sigma-Aldrich (St Louis, MO, USA), *Pfx*50 DNA polymerase was purchased from Invitrogen (Merelbeke, Belgium) and restriction enzymes were provided by New England Biolabs (Hertfordshire, UK). Polymerase chain reactions (PCR) were carried out with the Mastercycler Gradient from Eppendorf (Hamburg, Germany). Plasmid DNA purification was performed with the Nucleobond™ AX500 kit (Machery-Nagel, Düren, Germany) or the Plasmid mini kit I (Omega Bio-Tek, Doraville, GA, USA). DNA was sequenced with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Human thrombin, human plasmin and rabbit thrombomodulin were purchased from Sigma-Aldrich, Enzyme Research Labs (South Bend, UK) and American Diagnostica (Greenwich, CT, USA), respectively. H-D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), aprotinin, hippuryl-L-arginine and PTCI were obtained from Biomol Research Labs (Plymouth Meeting, PA, USA), Fluka (Buchs, Switzerland), Bachem (Bubendorf, Switzerland) and Calbiochem (La Jolla, CA, USA), respectively. Tissue-type plasminogen activator (Actilyse®) was a kind gift from Boehringer Ingelheim (Brussels, Belgium). Citrated plasma of 16 healthy individuals, collected with their written consent, was pooled for clot lysis experiments. Thrombin and plasmin substrates (S-2238 and S-2403) were obtained from Nodia/Chromogenix (Antwerp, Belgium).

Construction of the nanobody library and expression and purification of binders

An alpaca (*Vicugna pacos*) was immunized through seven subcutaneous injections, at weekly intervals, of 100 μg of a stable TAFI variant (TAFI-A^{147}-C^{305}-I^{325}-I^{329}) mixed with Gerbu adjuvant [39]. Blood was collected and peripheral blood lymphocytes were prepared with Lymphoprep (Axis-Shield, Norway). Construction of the VHH library was performed as described previously [40]. The phage-displayed library was used for two parallel pannings against another stable variant, TAFI-A^{147}-C^{305}-I^{325}-I^{329}-Y^{333}-Q^{335}, either in its proenzyme form or in its activated form, according to the protocol described by Conrath *et al.* [40]. Identification of positive clones was performed by ELISA and 'identical' clones were identified by sequencing. Then, the VHH genes of the positive clones were recloned in the pHEN6 expression vector using *Bst*EII and *Pst*I. The constructs were transformed in *Escherichia coli* WK6 cells and expression was obtained according to the procedure as described previously [40]. The periplasmic extract was dialyzed against 20 mmol L^{-1} Tris-HCl containing 0.5 mol L^{-1} NaCl (pH 7.9), loaded on a His-Trap HP column (GE Healthcare, Uppsala, Sweden) and bound proteins were eluted using an imidazole gradient (0–350 mmol L^{-1} imidazole in 20 mmol L^{-1} Tris–HCl, 0.5 mol L^{-1} NaCl, pH 7.9). The nanobody containing fractions were pooled and dialyzed against phosphate-buffered saline (PBS; 140 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 8 mmol L^{-1} Na_2HPO_4, 1.5 mmol L^{-1} KH_2PO_4; pH 7.4).

Evaluation of the overall inhibitory effect of the nanobodies on TAFI activation and TAFIa activity

The overall inhibitory effect was measured as described earlier with some minor modifications [26]. Purified TAFI

(45 nmol L^{-1}, concentration at activation step) in HEPES buffer (25 mmol L^{-1} HEPES, 137 mmol L^{-1} NaCl, 3.5 mmol L^{-1} KCl, + 0.1% BSA; pH 7.4) was incubated (10 min at 25 °C) with either buffer or nanobody (32-fold molar excess over TAFI or a concentration ranging from 0.5- to 32-fold) prior to activation by thrombin, thrombomodulin and $CaCl_2$ (concentrations at activation step: 20, 5 and 7.5 mmol L^{-1}, respectively). After 10 min at 25 °C, PPACK (concentration to stop the activation reaction: 37.5 μmol L^{-1}) and the substrate hippuryl-arginine (Hip-Arg; concentration during substrate conversion: 4 mmol L^{-1}) was added to the activation mixtures and substrate conversion was allowed to proceed for 10 min at 25 °C. Reaction mixtures (100 μL) were stopped by addition of 20 μL HCl (1 mol L^{-1}) followed by neutralization with 20 μL NaOH (1 mol L^{-1}) and buffered with 25 μL Na_2HPO_4 (1 mol L^{-1}; pH: 7.4). Subsequently, 6% cyanuric chloride, dissolved in 1,4-dioxane (30 μL), was added and the mixture was vortexed and centrifuged. The supernatant was transferred into a 96-well microtiter plate and the absorbance at 405 nm was measured. From the comparison of the enzymatic activity of TAFIa generated in the absence and in the presence of nanobody, the inhibiting capacity of the nanobody (Nb) was calculated and expressed as percentage inhibition $\{([OD]^{noNb} - [OD]^{withNb})/([OD]^{noNb}) \times 100 = \%$ inhibition$\}$. Reduced TAFIa activity as detected in this experimental setup can be caused either by interference with the activation mechanism or by direct interference with the TAFIa enzymatic activity.

A similar method was applied for the evaluation of the effect of the nanobodies on the plasmin-mediated activation, except for (i) the use of plasmin (concentration at activation step: 500 nmol L^{-1}) instead of thrombin/thrombomodulin (T/TM), (ii) addition of aprotinin (concentration to stop activation: 125 U mL^{-1}) and (iii) incubation with Hip-Arg for 25 min.

As thrombin is a very weak activator of TAFI and because a prolonged activation time is counteracted by the instability of TAFIa (resulting in TAFIa levels that are too low to allow a reliable quantitative evaluation of the nanobody effects) an adapted method was designed for the evaluation of the effect of the nanobodies on the thrombin-mediated activation, for example TAFI was replaced by TAFI-A^{147}-C^{305}-I^{325}-I^{329}-Y^{333}-Q^{335} (concentration at activation step: 45 nmol L^{-1}), thrombin was used at a higher concentration (concentration at activation step: 100 nmol L^{-1}) and no thrombomodulin was added. The activation reaction was allowed to proceed for 2 h. Substrate conversion (10 min) and activity measurement was performed as described above.

Evaluation of the effect of the nanobodies on the activation of TAFI to TAFIa

TAFI (concentration at activation step: 0.41 μmol L^{-1} in Tris buffer; 20 mmol L^{-1} Tris, 100 nmol L^{-1} NaCl, pH 7.4) was incubated with either buffer or nanobody (sixteenfold molar excess over TAFI) for 10 min at 37 °C prior to activation with thrombin, thrombomodulin and $CaCl_2$ (concentration at activation step: 23.1, 5.8 and 5.8 mmol L^{-1}, respectively). The reactions were stopped by adding PPACK (34.5 μmol L^{-1}) and sodium dodecyl sulfate (SDS) (1% final concentration) to the reaction mixtures followed by heating for 30 s at 100 °C. The generated fragments were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), using Phast-Gel™ gradient 10–15% gels, followed by silver staining.

Evaluation of the direct inhibitory effect of the nanobodies on TAFIa activity

To determine the direct inhibitory effect of the nanobodies on TAFIa activity, TAFI (concentration during activation: 45 nmol L^{-1}, in HEPES buffer) was activated by thrombin/thrombomodulin (concentration of thrombin and thrombomodulin during activation: 20 and 5 nmol L^{-1}, respectively) at 25 °C prior to incubation (10 min) with an equal volume of either buffer or nanobody (thirty-two-fold molar excess over TAFI or a concentration ranging from 0.5- to thirty-two-fold) as described earlier [26]. Quantification of the residual TAFIa was determined using the substrate Hip-Arg as described [26]. The percentage inhibition of TAFIa activity was calculated relative to TAFIa activity in the absence of nanobody.

Evaluation of the effect of nanobodies on the enzymatic properties of TAFIa

Hydrolysis of Hip-Arg by TAFIa was determined in the presence and absence of nanobody. TAFI (1 μmol L^{-1} in HEPES buffer) was activated by T/TM (20 and 80 nmol L^{-1}, respectively) for 10 min at 25 °C. Thrombin activity was stopped by addition of PPACK (30 μmol L^{-1}). Then nanobody was added (molar ratio Nb: TAFI of 0.5–1) and samples were incubated for 10 min at 25 °C. Subsequently the velocity of the substrate hydrolysis (Hip-Arg) at various concentrations (0.125–4 mmol L^{-1}) was monitored at 254 nm. The k_{cat} and K_M values were determined by non-linear regression of the data to the Michaelis–Menten equation (mean ± SD, $n \geq 4$) [39].

Evaluation of the effect of the nanobodies on the catalytic activity of TAFI

The catalytic activity of the zymogen was determined as described previously [41]. To determine the effect of the nanobodies on the zymogen activity, TAFI (concentration during substrate conversion: 0.119 μmol L^{-1} in HEPES buffer) was incubated for 10 min at 37 °C with either buffer or nanobody (thirty-two-fold molar excess over TAFI) prior to incubation with Hip-Arg (concentration during substrate conversion: 11.1 mmol L^{-1}). Substrate conversion was allowed to proceed for 4 h. Evaluation of the amount of converted substrate and quantitative evaluation of the effect of nanobody was carried out as described above.

Evaluation of the effect in an in vitro *clot lysis experiment*

Clot lysis was performed as described previously with some small modifications [42]. Pooled plasma (30% final concentration) was mixed with buffer, nanobody or PTCI (20 μg mL^{-1}, final concentration). The final concentration of nanobody was 2.14 μmol L^{-1}, resulting in a thirty-two-fold molar excess over TAFI assuming a concentration of 178 nmol L^{-1} TAFI in plasma. Alternatively, a nanobody concentration resulting in a nanobody/TAFI ratio ranging from 0.5- to thirty-two-fold was used. Exogenous thrombomodulin (1 nmol L^{-1}, final concentration) and tPA (90 pmol L^{-1}) was added to the plasma. After clot induction with $CaCl_2$ (10.6 mmol L^{-1}), the plate was incubated at 37 °C and read at 405 nm at 2-min intervals to determine the 50% clot lysis time, defined as the time from full clot formation to the midpoint of the maximal turbid to clear transition. Reduction of the clot lysis time was calculated relative to the reduction of clot lysis time in the absence (0% reduction) and presence of PTCI (20 μg mL^{-1}, final concentration; 100% reduction). Alternatively, this assay was carried out without the addition of exogenous thrombomodulin.

Another variant of this assay was performed by adding nanobody (or buffer or PTCI or MA-T12D11) after the clot was formed [26]. The volume of the added solution after clot formation was 20 μL (i.e. 20% of clot volume). The final concentration of all components was identical to that indicated above.

Affinity measurements

Affinity constants for the binding between nanobodies and TAFI were determined by surface plasmon resonance (SPR) analysis using the Biacore 3000 analytical system (Biacore AB, Uppsala, Sweden) equipped with a CM-5 sensor chip as described [26]. In brief, nanobodies were covalently coupled to approximately 1200 resonance units using a concentration of 5 μg mL^{-1} nanobody in acetate buffer (10 mmol L^{-1} pH 4.5). TAFI was diluted in HBS-EP buffer (Biacore) to concentrations between 5 and 200 nmol L^{-1} and injected at a flow rate of 30 μL per min. After each cycle, the chip was regenerated with 10 μL of 10 mmol L^{-1} glycine solution pH 1.5. Analysis of the association and dissociation phases was performed using the Biacore 3000 software (Langmuir binding, local fit). All experiments were performed at least three times independently.

Statistical analysis

Quantitative data were summarized by the mean and standard deviation. Statistical analyzes on enzyme kinetics of TAFIa were performed with one-way ANOVA.

For the sake of clarity, only values of inhibition exceeding 40%, as observed in the enzymatic assays (Table 1), were considered as relevant for classifying the nanobodies according to their functional properties. Using SPSS software and appropriate analysis by pairwise comparisons between the groups on the five inhibitory properties revealed significant differences ($P < 0.05$) between these groups and confirms the classification into eight groups (see Results).

Results

Identification of nanobodies against TAFI(a)

After immunizing an Alpaca with TAFI-A^{147}-C^{305}-I^{325}-I^{329}, we cloned its nanobody repertoire from 9×10^7 peripheral blood lymphocytes in a phage-display vector. Enrichment for antigen-specific clones was performed by two parallel pannings of this library against the stable variant, TAFI-A^{147}-C^{305}-I^{325}-I^{329}-Y^{333}-Q^{335}, either in its proenzyme form (three rounds of panning) or in its activated form (four rounds of panning). This resulted in the selection of 18 and 12 different nanobodies, respectively. Twenty-two out of these 30 nanobodies were found to exhibit inhibitory properties towards TAFI activation or TAFI(a) activity.

Nanobodies that resulted from panning against the proenzyme (intact TAFI) are denominated as 'Vhh-TAFI-i', whereas nanobodies selected through panning against the activated form are denominated 'Vhh-TAFI-a'. All nanobodies were produced in *E. coli* and purified by immobilized metal affinity chromatography.

Overall inhibitory effect of nanobodies towards TAFI

Addition of nanobody prior to activation of TAFI results in the possible detection of nanobodies interfering with either TAFI activation or TAFIa activity or both. This assay revealed that five nanobodies interfere with the thrombin/thrombomodulin mediated TAFI activation and/or TAFIa activity resulting in a 52%–100% inhibition at a thirty-two-fold molar excess (Table 1, column 3). Four of these nanobodies exhibited a strong inhibitory effect with values between 95% and 100% (group 1, 2 and 3), whereas one nanobody, Vhh-TAFI-i32, revealed only a moderate inhibition (52.3% ± 3.2%) (group 4). It should be noted that classification in groups (Table 1, column 3) is not solely based on the behaviour in this particular assay, but based on the results obtained with all assays.

Dose-response experiments were performed for the nanobodies with a strong inhibitory effect (group 1–3) and demonstrate that a maximal inhibitory effect is already observed at a 2-fold molar excess (Fig. 1A).

Nanobodies of group 1–4 were also able to inhibit thrombin-mediated TAFI activation and/or TAFIa activity, resulting in inhibitory properties between 62% and 99% (Table 1, column 4, group 1–4). In addition, five other nanobodies, that were not able to interfere with TAFI when activated by T/TM, did interfere with thrombin-mediated TAFI activation and/or TAFIa activity resulting in inhibitory values between 43% and 74% (Table 1, column 4, group 5 and 6). The nanobodies of group 6 did not have an effect on thrombin activity as evaluated with the chromogenic substrate S-2238 (data not shown) thereby excluding that the observed effect on

Table 1 Effect* of nanobodies on the enzymatic properties of TAFI(a), its activation and on clot lysis times

Nanobody	Group	T/TM-mediated activation	T-mediated activation	Plasmin-mediated activation	TAFIa activity	Zymogen activity	Profibrinolytic effect**
Vhh-TAFI-i83	1	**96 ± 0.3**	**87 ± 3**	**83 ± 2**	**88 ± 1**	**64 ± 4**	**83 ± 8**
Vhh-TAFI-a206	2	**96 ± 0.3**	**76 ± 3**	**67 ± 3**	**47 ± 3**	**52 ± 10**	**76 ± 7**
Vhh-TAFI-i108m	2	**96 ± 1**	**76 ± 4**	**77 ± 4**	**46 ± 2**	**42 ± 20**	**80 ± 8**
Vhh-TAFI-a204	3	**100 ± 1**	**99 ± 0.3**	**100 ± 1**	5.1 ± 3.7	**48 ± 11**	**100 ± 2**
Vhh-TAFI-i32	4	**52 ± 4**	**62 ± 3**	9.3 ± 8.6	1.7 ± 3.2	**56 ± 3**	33 ± 11
Vhh-TAFI-a190	5	16 ± 7	**43 ± 4**	**68 ± 3**	−11 ± 9	10 ± 18	21 ± 6
Vhh-TAFI-a52	6	19 ± 4	**45 ± 6**	36 ± 18	−6.2 ± 10.9	9.7 ± 16.6	14 ± 4
Vhh-TAFI-a104	6	3.2 ± 6.1	**44 ± 6**	−13 ± 10	−4.3 ± 12.0	14 ± 7	3.7 ± 1.6
Vhh-TAFI-a215	6	16 ± 3	**44 ± 7**	32 ± 8	−14 ± 7	16 ± 24	−1.0 ± 8.2
Vhh-TAFI-i64	6	3.5 ± 2.7	**74 ± 4**	−0.4 ± 7.1	7.7 ± 3.8	30 ± 11	5.7 ± 15.0
Vhh-TAFI-a200	7	10 ± 4	0.3 ± 1.5	**53 ± 6**	−14 ± 6	6.2 ± 14.9	34 ± 8
Vhh-TAFI-a263	7	9.5 ± 4.6	−13.4 ± 6.2	**56 ± 7**	2.1 ± 7.8	0.3 ± 17.0	36 ± 2
Vhh-TAFI-a306	7	12 ± 4	25 ± 7	**41 ± 9**	−7.3 ± 3.1	21 ± 24	22 ± 4
Vhh-TAFI-a327	7	13 ± 4	−7.9 ± 2.2	**57 ± 3**	−0.2 ± 10.2	9.9 ± 5.7	35 ± 5
Vhh-TAFI-i7	7	9.5 ± 3.5	−1.6 ± 1.2	**58 ± 5**	1.3 ± 4.3	12 ± 4	27 ± 3
Vhh-TAFI-i37	7	18 ± 1	−8.9 ± 1.9	**45 ± 2**	5.8 ± 4.6	12 ± 7	36 ± 8
Vhh-TAFI-i109	7	14 ± 3	−4.8 ± 12.2	**45 ± 8**	0.6 ± 3.9	2.2 ± 11.7	35 ± 3
Vhh-TAFI-i129	7	9.4 ± 4.0	−1 ± 13	**69 ± 3**	−2.5 ± 7.4	−2.2 ± 16.9	23 ± 0.02
Vhh-TAFI-i135	7	8.6 ± 1.6	−6.2 ± 5.8	**55 ± 3**	7.2 ± 5.0	6.8 ± 12.9	24 ± 4
Vhh-TAFI-i6	8	1.9 ± 3.4	30 ± 2	−2.0 ± 5.1	9.5 ± 8.9	**59 ± 6**	−12 ± 11
Vhh-TAFI-i46	8	4.9 ± 6.0	19 ± 4	−0.1 ± 6.1	−1.3 ± 3.1	**42 ± 6**	−0.2 ± 2.0
Vhh-TAFI-i66	8	5.4 ± 2.7	28 ± 1	−6.4 ± 7.7	7.6 ± 5.0	**41 ± 9**	−10 ± 6

*Expressed as percentage inhibition (see methods); mean ± SD, $n = 3$. **Reduction of clot lysis time, caused by the nanobody, expressed as percentage vs. the reduction caused by PTCI (100%) (see methods; mean ± SD, $n \geq 3$). Values of inhibition exceeding 40% are indicated in bold.

thrombin-mediated TAFI activation would be due to thrombin inhibition.

Evaluation of the effect on plasmin-mediated TAFI activation and/or TAFIa activity, revealed a strong inhibition (67%–100%) by the nanobodies of group 1, 2, 3 and 5. In addition, nine other nanobodies (Table 1, column 5, group 7), exhibiting virtually no effect on the T/TM- and T-mediated assays, exhibited a moderate but clearly distinct inhibition in the plasmin-mediated assay, with values between 41% and 69%. These nanobodies did not have an effect on plasmin activity as evaluated with the chromogenic substrate S-2403 (data not shown), thereby excluding that the observed effect on TAFI activation would be due to plasmin inhibition.

All other nanobodies (Table 1, column 3–5, group 8) exhibited no inhibitory effects (i.e. ≤40%), in this 'overall' assay, irrespective of the activator used.

Evaluation of the effect on the activation of TAFI to TAFIa

To examine the effect of the nanobodies on the activation of TAFI, the fragmentation products generated upon activation with T/TM (in the absence or presence of nanobody) were analyzed. In the absence of nanobody the typical fragmentation pattern was observed: disappearance of the 56-kDa proenzyme and generation of a 36- and 25-kDa fragment (Fig. 2, lane 2). This typical 25-kDa fragment is the subsequent cleavage product due to a secondary cleavage site at position Arg^{302} in conformationally inactivated TAFI [1]. All nanobodies of group 1, 2 and 3, (Vhh-TAFI-i83, Vhh-TAFI-a206, Vhh-TAFI-i108m, Vhh-TAFI-a204) hampered the conversion of TAFI (55 kDa) into TAFIa (36 kDa) virtually completely (Fig. 2, lane 3–6, respectively). Vhh-TAFI-i32 (group 4) moderately inhibited the conversion of TAFI into TAFIa (Fig. 2, lane 7). Vhh-TAFI-i37 (group 7) (Fig. 2, lane 8) and all other nanobodies of group 5 through to 8 (data not shown) did not affect the fragmentation pattern. All data obtained in this assay are in line with the respective properties observed in the overall inhibitory assay (T/TM).

Direct inhibitory effect on TAFIa activity

Addition of nanobody after activation of TAFI and subsequent evaluation of the enzymatic activity of TAFIa allowed the detection of nanobodies that interfere directly with TAFIa. Strikingly, only one nanobody (Vhh-TAFI-i83, group 1) revealed a strong direct inhibitory effect (88%) on the TAFIa activity. Two other nanobodies (Vhh-TAFI-i108m and Vhh-TAFI-a206, group 2) revealed a moderate inhibitory effect (46%) (Table 1, column 6). Dose-response experiments demonstrate that Vhh-TAFI-i83 reaches its maximal inhibitory effect at a 8-fold molar excess, whereas for Vhh-TAFI-i108m and Vhh-TAFI-a206 the maximal inhibitory effect was not yet reached at a thirty-two–fold molar excess (Fig. 1B).

From the absence of any inhibitory effect of Vhh-TAFI-a204 and Vhh-TAFI-i32 on the enzymatic activity it can be

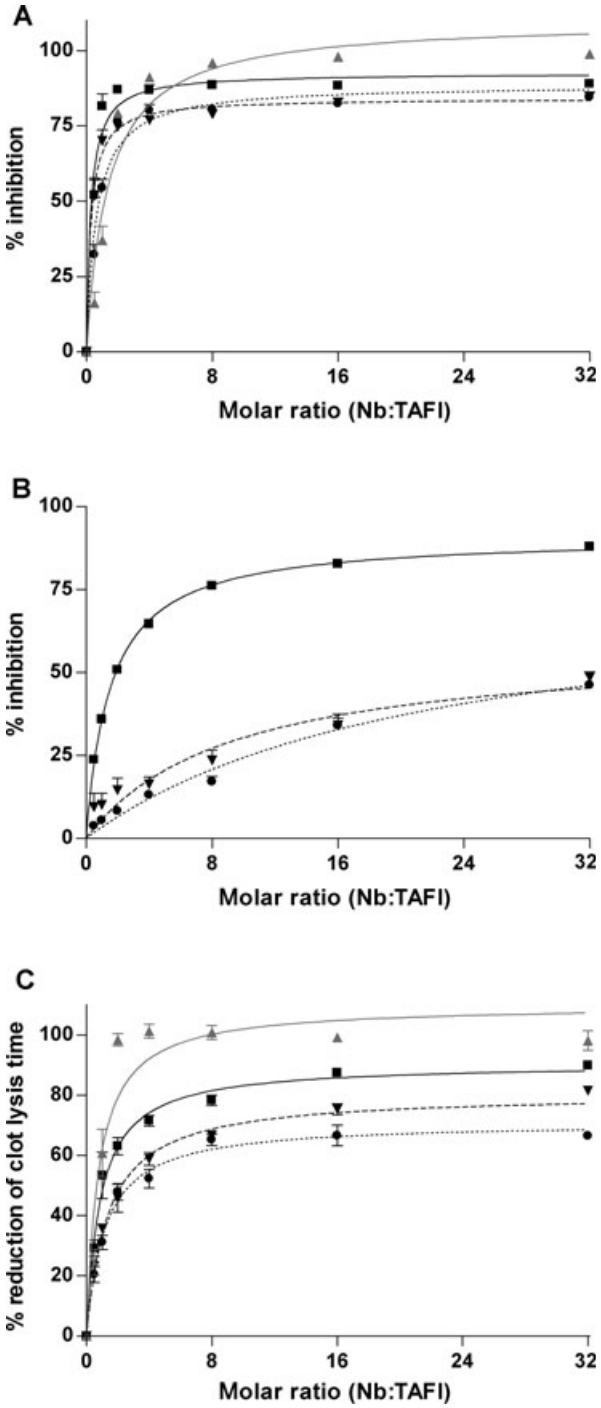

Fig. 1. Dose-dependent effect of Vhh-TAFI-i83 (■, black full line), Vhh-TAFI-a206 (▼, black dashed line), Vhh-TAFI-i108m (●, black dotted line) and Vhh-TAFI-a204 (▲, gray full line) on thrombin/thrombomodulin (T/TM)-mediated thrombin-activatable fibrinolysis inhibitor (TAFI) activation (A), activated thrombin-activatable fibrinolysis inhibitor (TAFIa) activity (B) and 50% clot lysis times (C). Percentage inhibition was calculated relative to TAFI activation or TAFIa activity in the absence of nanobody (= 100% activation/activity). Percentage reduction of 50% clot lysis time was calculated as percentage relative the reduction caused by potato tuber carboxypeptidase inhibitor (PTCI) (= 100% reduction). The results represent mean ± SD ($n \geq 3$).

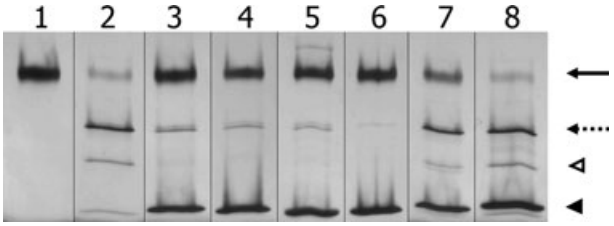

Fig. 2. Effect of nanobodies on fragmentation pattern of thrombin-activatable fibrinolysis inhibitor (TAFI) upon activation by thrombin/thrombomodulin (T/TM). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of TAFI, pre-incubated with buffer (lane 1and 2), or Vhh-TAFI-i83 (lane 3), Vhh-TAFI-a206 (lane 4), Vhh-TAFI-i108m (lane 5), Vhh-TAFI-a204 (lane 6), Vhh-TAFI-i32 (lane 7), Vhh-TAFI-i37 (lane 8) prior to activation with T/TM (lanes 2–8). Intact TAFI (56 kDa) is indicated by the arrow, activated thrombin-activatable fibrinolysis inhibitor (TAFIa) (36 kDa) by the dashed arrow, the 25 kDa fragment by the open arrowhead and the nanobody (15 kDa) by the arrowhead.

deduced that their effects observed in the overall inhibitory assay are exclusively due to an impairment of the activation step.

Effect of nanobodies on the enzymatic properties of TAFIa

Data of the hydrolysis of the substrate Hip-Arg by TAFIa in the absence and presence of nanobodies Vhh-TAFI-i83, Vhh-TAFI-i108m and Vhh-TAFI-a206 are shown in Fig. 3 and Table 2. Addition of Vhh-TAFI-i83, Vhh-TAFI-i108m and Vhh-TAFI-a206 resulted in a significant ($P < 0.005$) and dose-dependent decrease of the catalytic rate (k_{cat}). This is observed at very low inhibitor concentrations, in the same order of magnitude as the enzyme concentration, indicating that these nanobodies are tight-binding inhibitors [40].

Addition of Vhh-TAFIi83 and Vhh-TAFI-a206 had no effect on the K_M-value (Table 2). In contrast, addition of Vhh-TAFI-i108m resulted in a significant ($P < 0.0001$) and dose-dependent decrease of K_M. This suggests that the substrate may have a slightly higher affinity for the enzyme/inhibitor complex vs. free enzyme [43] and indirectly indicates a simultaneous binding of Vhh-TAFI-i108m and Hip-Arg to TAFIa.

Effect of nanobodies on the catalytic activity of the proenzyme TAFI

To evaluate the possible effect of the nanobodies on the catalytic activity of the zymogen, nanobodies were incubated with intact TAFI prior to incubation with Hip-Arg (and without activation with thrombin, T/TM or plasmin). Vhh-TAFI-i83, Vhh-TAFI-i108m, Vhh-TAFI-a206, Vhh-TAFI-a204 and Vhh-TAFI-i32 (group 1–4) exhibited a moderate inhibitory effect (42%–64%) on the zymogen activity (Table 1, column 7). In addition, three other nanobodies that exhibited no inhibition on TAFIa activity or TAFI activation (Table 1, column 7, group 8) revealed a moderate inhibition of the

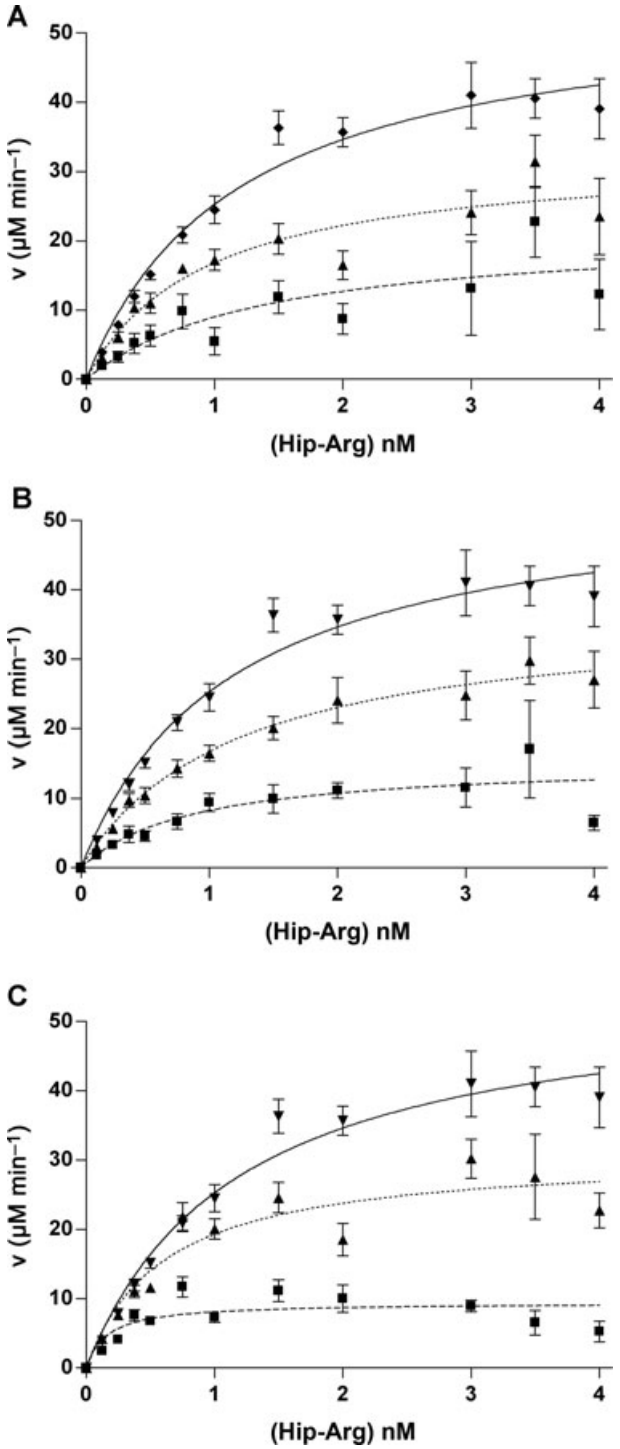

Fig. 3. Michaelis–Menten curves of Hip-Arg hydrolysis by activated thrombin-activatable fibrinolysis inhibitor (TAFIa) in the absence or presence of nanobody. (A) Vhh-TAFI-i83 at a molar ratio Nb:TAFI of 0 (▼, full line), 0.5 (▲, dotted line) and 0.75 (■, dashed line), (B) Vhh-TAFI-a206 at a molar ratio Nb:TAFI of 0 (▼, full line), 0.5 (▲, dotted line) and 1 (■, dashed line), (C) Vhh-TAFI-i108m at a molar ratio Nb:TAFI of 0 (▼, full line), 0.5 (▲, dotted line) and 1 (■, dashed line).

zymogen activity (41%–59%). All other nanobodies (group 5–7) exhibited no inhibitory effect in this assay (Table 1, column 7).

Effect of nanobodies in an in vitro *clot lysis experiment*

To test the functional effects of the nanobodies on clot lysis, plasma was preincubated with buffer (negative control), PTCI (reference) or nanobody. For the negative control and the reference, 50% clot lysis times of 91–120 and 54–65 min, respectively, were obtained in the presence of TM. In the absence of TM, 50% clot lysis times of 76–100 and 37–56 min were obtained for the negative control and the reference, respectively. Vhh-TAFI-i83, Vhh-TAFI-i108m, Vhh-TAFI-a206 and Vhh-TAFI-a204 (group 1–3) strongly reduced (76%–100%) the clot lysis times, whereas addition of nanobodies of group 4 (interfering moderately with T/TM- and with T-mediated activation and also with the intrinsic activity), group 5 (interfering predominantly with plasmin-mediated activation) and group 7 (interfering exclusively with plasmin-mediated activation), resulted in a minor reduction (21%–36%) of clot lysis times (Table 1). None of the other nanobodies (i.e. group 6 and 8) reduced clot lysis. Dose-response curves were performed for the nanobodies with the most pronounced effect on clot lysis (i.e. group 1–3). These data demonstrate that the maximal profibrinolytic effect is virtually reached at a 2- to 4-fold molar excess (Fig. 1C).

In the above-mentioned typical clot lysis experiments, TAFI inhibitors were added prior to clot formation. To evaluate the value of a TAFI inhibitor added after clot formation and the effect of the size of the inhibitor under those conditions, the nanobody Vhh-TAFI-a204 and the inhibitory monoclonal antibody MA-T12D11, added after clot formation, were subjected to a comparative evaluation. Addition of Vhh-TAFI-a204 after clot formation still yielded a reduction of the clot lysis time of 54% ± 5% (vs. 99% when added prior to clot formation, Table 1*)*, whereas the inhibitory monoclonal antibody MA-T12D11 yielded a reduction of only 21% ± 8% (Fig. 4) (vs. 92%–98% when added prior to clot formation [26,44]).

Thrombomodulin is a strong accelerator of TAFI activation. The presence of exogenously added TM in a typical clot lysis experiment could, therefore, obscure effects of inhibitory nanobodies that do not or only moderately interfere with T/TM-mediated activation (group 4, 5, 6 and 7) or interfere exclusively with the zymogen activity (group 8). Therefore, these nanobodies were evaluated in a clot lysis assay to which no exogenous thrombomodulin was added. Nanobody Vhh-TAFI-i32 (moderate interference with T/TM- and T-mediated activation and with zymogen activity, group 4), Vhh-TAFI-a190 (only interference with T- and with plasmin-mediated activation, group 5) and nanobodies from group 7 (only interference with plasmin-mediated activation) resulted in a 2-fold increased reduction of clot lysis time in the absence of exogenously added TM compared with that observed in the presence (45%–59% reduction vs. 21%–36% reduction, respectively). The effect observed with nanobodies of group 6 (exclusive interference with T-mediated activation) and of group 8 (mainly interfering with zymogen activity) was virtually independent of the absence or presence of exogenously

Table 2 Enzyme kinetics of Hip-Arg hydrolysis by TAFIa in the absence and presence of Vhh-TAFI-i83, Vhh-TAFI-a206 and Vhh-TAFI-i108m

	Molar ratio (Nb: TAFI)	K_M (mmol L^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
	0	1.21 ± 0.26	12.5 ± 2.04	10.6 ± 1.90
Vhh-TAFI-i83	0.5	0.881 ± 0.435	7.17 ± 2.76*	8.48 ± 0.93**
	0.75	1.19 ± 0.79	5.05 ± 3.49*	4.67 ± 1.96**
Vhh-TAFI-a206	0.5	1.41 ± 0.864	8.98 ± 3.93***	6.94 ± 1.88***
	1	0.896 ± 0.286	3.42 ± 1.55***	3.98 ± 1.58***
Vhh-TAFI-i108m	0.5	0.681 ± 0.279***	7.20 ± 1.83***	11.6 ± 2.95
	1	0.192 ± 0.119***	2.12 ± 0.37***	16.1 ± 10.2

Mean ± SD; $n \geq 4$. *$P < 0.002$, **$P < 0.0005$ and ***$P < 0.0001$ (one-way ANOVA).

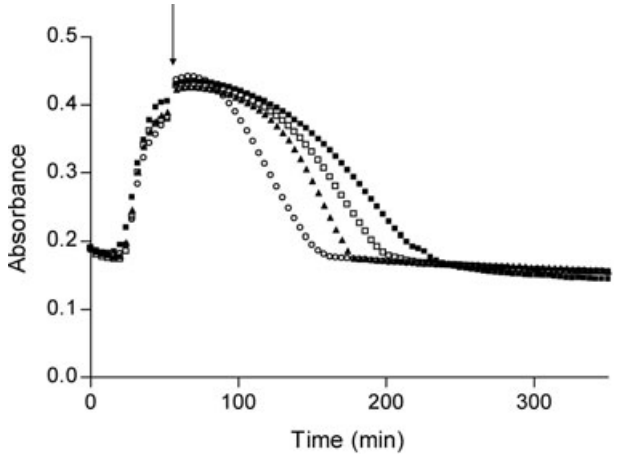

Fig. 4. Effect of the size of thrombin-activatable fibrinolysis inhibitor (TAFI) inhibitor (added after clot formation) on clot lysis. The arrow indicates the time point of addition of potato tuber carboxypeptidase inhibitor (PTCI) (○), Vhh-TAFI-a204 (▲), MA-T12D11 (□) or buffer (■).

added TM (−21%–20% vs. −12%–14%). The profibrinolytic effect of Vhh-TAFI-a204, included as a positive control, was fully conserved in the absence of exogenously added TM (101% ± 6% vs. 99.5% ± 2.0% in the presence of TM).

Affinity of nanobodies towards TAFI

Evaluation of the binding between TAFI and some selected nanobodies was performed by surface plasmon resonance analysis. A high affinity for TAFI (K_A–values between 1 and 5 nM^{-1}) was observed for the nanobodies of group 1, 2 and 3 (Table 3). Nanobodies of group 4, 5, 7 and 8,revealed a lower affinity towards TAFI (K_A–values between 3 and 24 nM^{-1}), mainly caused by high dissociation kinetics (*kd* in the order of 10^{-2} per seconds), except for Vhh-TAFI-i6, for which the lower affinity is the consequence of both a decreased association and dissociation kinetics. For a yet unknown reason no detectable binding was observed for Vhh-TAFI-i64 in these experiments; however, binding of Vhh-TAFI-i64 to TAFI was confirmed by ELISA (data not shown).

Discussion

TAFI hampers fibrinolysis by removing carboxy-terminal lysines from partially degraded fibrin. Removal of the lysine residues, a cofactor in the formation of the ternary complex of plasminogen and t-PA, results in a decreased plasmin formation and an inhibition of clot lysis. This function of TAFI makes it an attractive target for the development of new fibrinolytic drugs. Moreover, current established thrombolytic treatment with new generation plasminogen activators did not appear to circumvent the several side effects of tPA so major bleedings stays a serious complication of thrombolytic therapy [45]. Coadministration of a TAFI inhibitor might reduce complications and improve thrombolytic efficacy [13,14]. To date, several TAFI inhibitors have been generated [12,16,23,25,26,46,47]; however, small synthetic inhibitors often deal with a lack of specificity [48]. On the other hand, therapeutic use of monoclonal antibodies can cause immunogenicity problems. Nanobodies are the smallest antigen-binding antibody fragments and have superior features, such as high solubility and high stability because they are natural monomeric antigen binders [49]. It was previously demonstrated that nanobodies are often very potent enzyme inhibitors [40]. Therefore, and as a novel approach for the development of TAFI(a) inhibitors, we generated nanobodies.

Indeed, after immunization and panning with stable variants of TAFI, a panel of 22 inhibitory nanobodies was obtained. Importantly, this panel represents a wide diversity in modes of action of interference with the TAFI properties. These 22 nanobodies could be classified into eight different categories: (i) interfering with all mechanisms of activation, with the TAFIa activity and with the zymogen activity; (ii) interfering with all mechanisms of activation and only moderately with TAFIa activity and zymogen activity; (iii) interfering with all mechanisms of activation and moderately with zymogen activity but not with TAFIa activity; (iv) moderately interfering with T/TM- and T-mediated TAFI activation and zymogen activity but not with plasmin-mediated TAFI activation and TAFIa activity; (v) moderately interfering with T- and plasmin-mediated TAFI activation but not with T/TM-mediated TAFI activation, TAFIa activity and zymogen activity; (vi) exclusively interfering with T-mediated TAFI activation; (vii) exclusively interfering with plasmin-mediated TAFI activation; and (viii) exclusively interfering with zymogen activity.

Surprisingly, one nanobody, Vhh-TAFI-i83, strongly inhibits the activation of TAFI, for example preventing the proteolytic cleavage of the Arg^{92}-Ala^{93} bound, as well as the carboxypeptidase activity of activated TAFI (TAFIa), for

Table 3 Binding parameters for nanobodies towards TAFI

Nanobody	Group	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_A (M^{-1})
Vhh-TAFI-i83	1	$2.50 \pm 0.20 \times 10^5$	$4.74 \pm 0.04 \times 10^{-5}$	$5.31 \pm 1.19 \times 10^9$
Vhh-TAFI-i108m	2	$2.98 \pm 0.07 \times 10^5$	$1.67 \pm 0.03 \times 10^{-4}$	$1.95 \pm 0.46 \times 10^9$
Vhh-TAFI-a206	2	$1.70 \pm 0.21 \times 10^5$	$1.76 \pm 0.03 \times 10^{-4}$	$9.80 \pm 2.48 \times 10^8$
Vhh-TAFI-a204	3	$1.31 \pm 0.28 \times 10^6$	$9.23 \pm 0.20 \times 10^{-4}$	$1.42 \pm 0.68 \times 10^9$
Vhh-TAFI-i32	4	$1.18 \pm 0.19 \times 10^5$	$3.50 \pm 0.51 \times 10^{-2}$	$3.24 \pm 3.15 \times 10^6$
Vhh-TAFI-a190	5	$2.88 \pm 0.35 \times 10^5$	$1.58 \pm 0.06 \times 10^{-2}$	$1.72 \pm 1.03 \times 10^7$
Vhh-TAFI-i64	6	NB	NB	NB
Vhh-TAFI-a327	7	$2.88 \pm 0.90 \times 10^5$	$1.18 \pm 0.09 \times 10^{-2}$	$2.40 \pm 1.44 \times 10^7$
Vhh-TAFI-i129	7	$3.35 \pm 1.20 \times 10^5$	$2.03 \pm 0.13 \times 10^{-2}$	$1.82 \pm 1.13 \times 10^7$
Vhh-TAFI-i6	8	$4.10 \pm 0.62 \times 10^4$	$2.34 \pm 0.17 \times 10^{-3}$	$1.79 \pm 0.58 \times 10^7$

Mean ± SD; $n = 3$. NB, no binding; $K_A < 10^6$ M^{-1}.

example interfering with the residues of the enzymatic pocket (residues implicated in catalysis: Arg^{217} and Glu^{363}; in substrate binding: Arg^{235}, Tyr^{341} and Asn^{234}; in zinc binding: His^{159}, Glu^{162} and His^{288}). As Vhh-TAFI-i83 also interferes with the zymogen activity it is most likely that this nanobody binds directly into the catalytic cleft of TAFI(a). We do suggest that its effect on the activation is due to a subsequently induced conformational change/allosteric modulation of the region comprising the Arg^{92}-Ala^{93} bound. This hypothesis is also further substantiated by the observation that Vhh-TAFI-i83 impairs the activation irrespective of the activator used. Not surprisingly, the 'multi-inhibitory' properties of Vhh-TAFI-i83, together with its high affinity ($K_A = 5 \times 10^9$ M^{-1}) results in a strong profibrinolytic effect in clot lysis. To our knowledge, this is the first TAFI inhibitor that exhibit strong inhibitory properties towards the various modes of TAFI activation, as well as towards TAFIa activity and the zymogen activity.

Analysis of the enzyme kinetics of Hip-Arg hydrolysis by TAFIa in the absence and presence of Vhh-TAFI-i83, Vhh-TAFI-a206 and Vhh-TAFI-i108m (nanobodies that inhibit TAFIa activity) reveals that these nanobodies are tight-binding inhibitors, as has been reported for other nanobodies [40]. This is compatible with their high affinity as observed by surface plasmon resonance analysis. The effect of Vhh-TAFI-i108m on K_M suggests that this nanobody exerts its inhibitory effect through a different mechanism compared with that of Vhh-TAFI-i83 and Vhh-TAFI-a206.

In the currently used clot lysis model, four nanobodies exerted a strong acceleration of the lysis time, comparable to that of PTCI, indicating a complete knock-out of TAFI(a). It should be noted that this functional model is strongly TM dependent and yields data (Table 1) that are in line with the observation that these four nanobodies are very potent inhibitors of the T/TM-mediated TAFI activation (Table 1). On the other hand all other nanobodies with virtually no effect on clot lysis do not interfere with the T/TM-mediated activation. As TM accelerates the T-mediated TAFI activation one thousand two hundred and fifty-fold [5,47], it could be expected that in clot lysis experiments performed in the presence of TM, the latter nanobodies had no effect. However, comparison of the effects on clot lysis in the absence of exogenously added TM, observed for nanobodies of group 5 and 7 (no effect on T/TM mediated activation but a pronounced effect on plasmin-mediated activation) with those observed for nanobodies of group 6 (only a pronounced effect on T-mediated activation, no effect on T/TM- nor on plasmin-mediated activation), is strongly suggestive for a significant role of plasmin-mediated activation of TAFI during clot lysis. This is indicative for a dual role of plasmin during fibrinolysis: fibrinolytic due to its fibrin degradation properties on the one hand, but antifibrinolytic due to its capability to activate TAFI. The relative contribution of the various TAFI activators *in vivo* is currently not known. Our results demonstrate that nanobodies which exclusively interfere with plasmin-mediated activation (i.e. group 7) may constitute useful tools to further unravel the relative contribution of each of the physiological activators. In this respect it is important to realize that TAFI is not only implicated in fibrinolysis but also plays a role in inflammation, cell migration and blood pressure [9–12], processes in which plasmin- rather than T/TM-mediated activation may be involved.

To the best of our knowledge this is the first report identifying TAFI inhibitors that interfere exclusively with the TAFI zymogen activity. Even although there is a report suggesting a physiological role for TAFI zymogen activity [50], our data are in line with the suggestion that TAFI zymogen activity does not play a major role in fibrinolysis [51] as these nanobodies have no or no pronounced effect on clot lysis experiments, neither in the presence nor in the absence of TM.

An additional, important advantage for potential therapeutic application of nanobodies is their ten-fold smaller size compared with conventional monoclonal antibodies. We, therefore, anticipated that TAFI inhibitory nanobodies may penetrate the clot more efficiently than TAFI inhibitory monoclonal antibodies. Indeed, whereas Vhh-TAFI-a204 and MA-T12D11 have a similar affinity (K_A 1.4×10^9 and 5×10^9 M^{-1}, respectively) and affect clot lysis to a similar extent when added prior to clot formation, the profibrinolytic effect of the nanobody was only 2-fold reduced when added after clot formation, in contrast to the effect of MA-T12D11 that was 4-fold reduced. These data strongly suggest that the inhibitory nanobodies do better penetrate into the clot and as such may constitute a better therapeutic agent for for example adjunctive therapy during thrombolytic treatment.

Acknowledgement

The authors thank G. Compernolle for excellent technical assistance. This study was financial supported in part by grant OT/06/66 of the Research Fund K. U. Leuven, and grant G.0833.09 from the Fund for Scientific Research-Flanders

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Bajzar L, Manuel R, Nesheim ME. Purification and characterization of TAFI, a thrombin-activatable fibrinolysis inhibitor. *J Biol Chem* 1995; **270**: 14477–84.
2 Hendriks D, Scharpe S, Vansande M, Lommaert MP. Characterization of a carboxypeptidase in human-serum destinct from carboxypeptidase-N. *J Clin Chem Clin Biochem* 1989; **27**: 277–85.
3 Eaton DL, Malloy BE, Tsai SP, Henzel W, Drayna D. Isolation, molecular-cloning, and partial characterization of a novel carboxypeptidase-B from human plasma. *J Biol Chem* 1991; **266**: 21833–8.
4 Wang W, Hendriks DF, Scharpe SS. Carboxypeptidase-U, a plasma carboxypeptidase with high-affinity for plasminogen. *J Biol Chem* 1994; **269**: 15937–44.
5 Bajzar L, Morser J, Nesheim M. TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. *J Biol Chem* 1996; **271**: 16603–8.
6 Wang W, Boffa PB, Bajzar L, Walker JB, Nesheim ME. A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor. *J Biol Chem* 1998; **273**: 27176–81.
7 van Tilburg NH, Rosendaal FR, Bertina RM. Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis. *Blood* 2000; **95**: 2855–9.
8 Silveira A, Schatteman K, Goossens F, Moor E, Scharpé S, Strömqvist M, Hendriks D, Hamsten A. Plasma procarboxypeptidase U in men with symptomatic coronary artery disease. *Thromb Haemost* 2000; **84**: 364–8.
9 Campbell WD, Lazoura E, Okada N, Okada H. Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N. *Microbiol Immunol* 2002; **46**: 131–4.
10 Myles T, Nishimura T, Yun TH, Nagashima M, Morser J, Patterson AJ, Pearl RG, Leung LLK. Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation. *J Biol Chem* 2003; **278**: 51059–67.
11 te Velde EA, Wagenaar GTM, Reijerkerk A, Roose-Girma M, Rinkes I, Voest EE, Bouma BN, Gebbink M, Meijers JCM. Impaired healing of cutaneous wounds and colonic anastomoses in mice lacking thrombin-activatable fibrinolysis inhibitor. *J Thromb Haemost* 2003; **1**: 2087–96.
12 Asai S, Sato T, Tada T, Miyamoto T, Kimbara N, Motoyama N, Okada H, Okada N. Absence of procarboxypeptidase R induces complement-mediated lethal inflammation in lipopolysaccharide primed mice. *J Immunol* 2004; **173**: 4669–74.
13 Nagashima M, Werner M, Wang M, Zhao L, Light DR, Pagila R, Morser J, Verhallen P. An inhibitor of activated thrombin-activatable fibrinolysis inhibitor potentiates tissue-type plasminogen activator-induced thrombolysis in a rabbit jugular vein thrombolysis model. *Thromb Res* 2000; **98**: 333–42.
14 Klement P, Liao P, Bajzar L. A novel approach to arterial thrombolysis. *Blood* 1999; **94**: 2735–43.
15 Mao SS, Holahan MA, Bailey C, Wu GX, Colussi D, Carroll SS, Cook JJ. Demonstration of enhanced endogenous fibrinolysis in thrombin activatable fibrinolysis inhibitor-deficient mice. *Blood Coagul Fibrinolysis* 2005; **16**: 407–15.
16 Wang Y-X, da Cunha V, Vincelette J, Zhao L, Nagashima M, Kawai K, Yuan S, Emayan K, Islam I, Hosoya J, Sullivan ME, Dole WP, Morser J, Buckman BO, Vergona R. A novel inhibitor of activated thrombin activatable fibrinolysis inhibitor (TAFIa) – part II: enhancement of both exogenous and endogenous fibrinolysis in animal models of thrombosis. *Thromb Haemost* 2007; **97**: 54–61.
17 Schneider M, Boffa M, Stewart R, Rahman M, Koschinsky M, Nesheim M. Two naturally occurring variants of TAFI (Thr-325 and Il-325) differ substantially with respect to thermal stability and antifibrinolytic activity of the enzyme. *J Biol Chem* 2002; **277**: 1021–30.
18 Ceresa E, Peeters M, Declerck PJ, Gils A. Announcing a TAFIa mutant with a 180-fold increased half-life and concomitantly a strongly increased antifibrinolytic potential. *J Thromb Haemost* 2007; **5**: 418–20.
19 Hendriks D, Wang W, Scharpe S, Lommaert MP, Vansande M. Purification and characterization of a new arginine carboxypeptidase in human serum. *Biochim Biophys Acta* 1990; **1034**: 86–92.
20 Mao SS, Colussi D, Bailey CM, Bosserman M, Burlein C, Gardell SJ, Carroll SS. Electrochemiluminescence assay for basic carboxypeptidases: inhibition of basic carboxypeptidases and activation of thrombin-activatable fibrinolysis inhibitor. *Anal Biochem* 2003; **319**: 159–70.
21 Lazoura E, Campbell W, Yamaguchi Y, Kato K, Okada N, Okada H. Rational structure-based design of a novel carboxypeptidase R inhibitor. *Chem Biol* 2002; **9**: 1129–39.
22 Barrow JC, Nantermet PG, Stauffer SR, Ngo PL, Steinbeiser MA, Mao SS, Carroll SS, Bailey C, Colussi D, Bosserman M, Burlein C, Cook JJ, Sitko G, Tiller PR, Miller-Stein CM, Rose M, McMasters DR, Vacca JP, Selnick HG. Synthesis and evaluation of imidazole acetic acid inhibitors of activated thrombin-activatable fibrinolysis inhibitor as novel antithrombotics. *J Med Chem* 2003; **46**: 5294–7.
23 Polla MO, Tottie L, Norden C, Linschoten M, Musil D, Trumpp-Kallmeyer S, Aukrust IR, Ringom R, Holm KH, Neset SM, Sandberg M, Thurmond J, Yu P, Hategan G, Anderson H. Design and synthesis of potent, orally active, inhibitors of carboxypeptidase U (TAFIa). *Bioorg Med Chem* 2004; **12**: 1151–75.
24 Suzuki K, Muto Y, Fushihara K, Kanemoto K, Iida H, Sato E, Kikuchi C, Matsushima T, Kato E, Nomoto M, Yoshioka S, Ishii H. Enhancement of fibrinolysis by EF6265[(S)-7-amino-2-[[[(R)-2-methyl-1-(3-phenylpropanoylamino)propyl]hy droxyphosphinoyl]methyl]heptanoic acid], a specific inhibitor of plasma carboxypeptidase B. *J Pharmacol Exp Ther* 2004; **309**: 607–15.
25 Bunnage ME, Blagg J, Steele J, Owen DR, Allerton C, MeElroy AB, Miller D, Ringer T, Butcher K, Beaumont K, Evans K, Gray AJ, Holland SJ, Feeder N, Moore RS, Brown DG. Discovery of potent & selective inhibitors of activated thrombin-activatable fibrinolysis inhibitor for the treatment of thrombosis. *J Med Chem* 2007; **50**: 6095–103.
26 Gils A, Ceresa E, Macovei AM, Marx PF, Peeters M, Compernolle G, Declerck PJ. Modulation of TAFI function through different pathways – implications for the development of TAFI inhibitors. *J Thromb Haemost* 2005; **3**: 2745–53.
27 Bajzar L, Nesheim ME, Tracy PB. The profibrinolytic effect of activated protein C in clots formed from plasma is TAFI-dependent. *Blood* 1996; **88**: 2093–100.
28 Hillmayer K, Vancraenenbroeck R, De Maeyer M, Compernolle G, Declerck PJ, Gils A. Discovery of novel mechanisms and molecular targets for the inhibition of activated thrombin activatable fibrinolysis inhibitor. *J Thromb Haemost* 2008; **6**: 1892–9.
29 Huston JS, Levinson D, Mudgetthunter M, Tai MS, Novotny J, Margolies MN, Ridge RJ, Bruccoleri RE, Haber E, Crea R, Oppermann H. Protein engineering of antibody-binding sites – recovery of specific activity in an anti-digoxin single-chain FV analog produced in Escherichia coli. *Proc Natl Acad Sci USA* 1988; **85**: 5879–83.
30 Presta LG. Molecular engineering and design of therapeutic antibodies. *Curr Opin Immunol* 2008; **20**: 460–70.
31 Mallender WD, Carrero J, Voss EW. Comparative properties of the single chain antibody and Fv derivatives of mAb 4-4-20 – Relationship

between interdomain interactions and the high affinity for fluorescein ligand. *J Biol Chem* 1996; **271**: 5338–46.
32 Whitlow M, Bell BA, Feng SL, Filpula D, Hardman KD, Hubert SL, Rollence ML, Wood JF, Schott ME, Milenic DE, Yokota T, Schlom J. An improved linker for single-chain FV with reduced aggregation and enhanced proteolytic stability. *Protein Eng* 1993; **6**: 989–95.
33 Hamerscasterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R. Naturally-occurring antibodies devoid of light-chains. *Nature* 1993; **363**: 446–8.
34 Muyldermans S, Atarhouch T, Saldanha J, Barbosa J, Hamers R. Sequence and structure of VH domain from naturally occuring camel heavy-chain immunoglobulins lacking light chains. *Protein Eng* 1994; **7**: 1129–35.
35 Muyldermans S. Single domain camel antibodies: current status. *J Biotechnol* 2001; **74**: 227–302.
36 Ghahroudi MA, Desmyter A, Wyns L, Hamers R, Muyldermans S. Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett* 1997; **414**: 521–6.
37 Rahbarizadeh F, Rasaee MJ, Forouzandeh M, Allameh AA. Over expression of anti-MUC1 single-domain antibody fragments in the yeast Pichia pastoris. *Mol Immunol* 2006; **43**: 426–35.
38 Vu KB, Ghahroudi MA, Wyns L, Muyldermans S. Comparison of llama V-H sequences from conventional and heavy chain antibodies. *Mol Immunol* 1997; **34**: 1121–31.
39 Ceresa E, van de Borne K, Peeters M, Lijnen HR, Declerck PJ, Gils A. Generation of a stable activated thrombin activable fibrinolysis inhibitor variant. *J Biol Chem* 2006; **281**: 15878–83.
40 Conrath KE, Lauwereys M, Galleni M, Matagne A, Frere JM, Kinne J, Wyns L, Muyldermans S. Beta-lactamase inhibitors derived from single-domain antibody fragments elicited in the Camelidae. *Antimicrob Agents Chemother* 2001; **45**: 2807–12.
41 Buelens K, Hillmayer K, Compernolle G, Declerck PJ, Gils A. Biochemical importance of glycosylation in thrombin activatable fibrinolysis inhibitor. *Circ Res* 2008; **102**: 295–301.
42 Develter J, Booth NA, Declerck PJ, Gils A. Bispecific targeting of thrombin activatable fibrinolysis inhibitor and plasminogen activator inhibitor-1 by a heterodimer diabody. *J Thromb Haemost* 2008; **6**: 1884–91.
43 Webb JL. *Enzyme and Metabolic Inhibitors. Volume I: General Principles of Inhibition*. New York and London: Academic Press, 1963.
44 Develter J, Dewilde M, Gils A, Declerck PJ. Comparative study of inhibitory antibody derivatives towards thrombin activatable fibrinolysis inhibitor. *Thromb Haemost* 2009; **102**: 69–75.
45 Khan IA, Gowda RM. Clinical perspectives and therapeutics of thrombolysis. *Int J Cardiol* 2003; **91**: 115–27.
46 Do YH, Gifford-Moore DS, Beight DW, Rathnachalam R, Klimkowski VJ, Warshawsky AM, Lu DS. Inhibition of thrombin activatable fibrinolysis inhibitor by cysteine derivatives. *Thromb Res* 2005; **116**: 265–71.
47 Binette TM, Taylor FB, Peer G, Bajzar L. Thrombin-thrombomodulin connects coagulation and fibrinolysis: more than an *in vitro* phenomenon. *Blood* 2007; **110**: 3168–75.
48 Willemse JL, Heylen E, Nesheim ME, Hendriks DF. Carboxypeptidase U (TAFIa): a new drug target for fibrinolytic therapy? *J Thromb Haemost* 2009; **7**: 1962–71.
49 Dumoulin M, Conrath K, Van Meirhaeghe A, Meersman F, Heremans K, Frenken LGJ, Muyldermans S, Wyns L, Matagne A. Single-domain antibody fragments with high conformational stability. *Protein Sci* 2002; **11**: 500–15.
50 Valnickova Z, Thogersen IB, Potempa J, Enghild JJ. Thrombin-activable fibrinolysis inhibitor (TAFI) zymogen is an active carboxypeptidase. *J Biol Chem* 2007; **282**: 3066–76.
51 Foley JH, Kim P, Nesheim ME. Thrombin-activable fibrinolysis inhibitor zymogen does not play a significant role in the attenuation of fibrinolysis. *J Biol Chem* 2008; **283**: 8863–7.